REMARKS

The specification has been amended to accurately refer to multiple-panel figures included with the formal drawings submitted herewith. No new matter has been added.

The Examiner has required restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 40-53, drawn to a method of regulating homeostasis of a cell comprising contacting the cell with a compound that regulates the activity of a MEKK protein, classified in class 435, subclass 7.1 for example.

II. Claim 53-64, drawn to a method for regulating apoptosis of a cell comprising contacting the cell with a compound that regulates the activity of a MEKK protein, classified in class 435, subclass 7.1 for example.

Applicant directs the Examiner's attention to the following discrepancies in the instant Office Action. On Form PTO-362, the Examiner has indicated that claims 41-64 are pending in the instant application. At page 2 of the Office Action, the Examiner restricts the claims of the invention (Claims 40-64) to one of two groups. Notably, claim 40 was left out of the recitation of the claims pending in the instant application on Form PTO-362. Moreover, the Examiner has required restriction of the invention to one of two groups of claims, Group I (Claims 40-53) and Group II (Claims 53-64). Notably, Claim 53 is included in both groups. As claim 53 is directed to a method of modulating apoptosis, Applicant shall assume that Claim 53 is properly included in Group II, only. Should the Examiner have comment to the contrary, it is respectfully requested that such comment be included in the next Office Action.

Applicant hereby elects Group II, Claims 53-64, drawn to methods for regulating apoptosis, for prosecution in the present application, without traverse.

The Examiner further requires restriction of the invention under 35 U.S.C. 121 to "a single nucleic acid or polypeptide", *i.e.*, a nucleic acid derived from SEQ ID NO:1, 3, 5, 7, 9,

11 or 13, or a polypeptide derived from SEQ ID NO:2, 4, 6, 8, 10. 12 or 14. The Examiner states that the recitation of multiple nucleic acids or polypeptides in the claims "constitutes recitation of an implied, mis-joined Markush group that contains multiple distinct inventions". The Examiner further states that "[e]ach of the different nucleic acids/polypeptides and methods of use are independent and distinct because no common structural or functional properties are shared". Quite contrary to the Examiner's assertion, the MEKK polynucleotides or polypeptides of the invention share significant structural and functional properties. In particular, the MEKK polypeptides of the invention (and nucleic acids encoding them) constitute a family of proteins sharing a conserved domain structure, the polypeptides each consisting of an N-terminal regulatory domain and a C-terminal kinase domain. Moreover, the family members share significant sequence identity, in particular within their enzymatically-active C-terminal kinase domains. Family members share functional activity as well, having enzymatic activity, *i.e.*, kinase activity, on known MEKK substrates. As the MEKK family members share core structure and activity, Applicant submits that polypeptides and nucleic acids are appropriately included in Markush groups, as indicated in the pending claims. Applicants submit that the respective MEKK family members are patentably distinct for the purposes of a species election, *i.e.*, for search purposes but traverses the requirement for election under 35 U.S.C. 121.

However, in order to be fully responsive, Applicant elects MEKK1 (*i.e*, SEQ ID NO: 1, 2, 3 and 4) for prosecution in the instant application.

Claims 40-64 are pending in the instant application. Non-elected claims 40-52 have been canceled without prejudice. No new claims have been added. Accordingly, claims 53-64 will be pending upon entry of the instant claim amendments.

Attached hereto is Appendix A, captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE**". The attached Appendix includes a marked-up version of the changes made to the specification by current amendment.

Cancellation of the claims was done to expedite prosecution of the instant application. Applicant reserves the right to pursue the claims as originally filed in this or a separate application(s). Applicant respectfully requests entry of the above specification amendments.

SUMMARY

If a telephone conversation with Applicant's Attorney would expedite the prosecution of the above-identified application, the examiner is urged to call Applicant's Attorney at (617) 227-7400.

Respectfully submitted,

Debra J. Milasincic
Registration No. 46,931
Attorney for Applicant

LAHIVE & COCKFIELD, LLP
60 State Street
Boston, MA 02109
Tel. (617) 227-7400
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APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

The paragraph beginning at page 8, line 21, has been replaced with the following rewritten paragraph:

[Figure 14] Figure 14A-B is a schematic representation of MEKK regulation of c-Myc controlled transcription.

The paragraph beginning at page 8, line 27, has been replaced with the following rewritten paragraph:

[Figure 16. Induction] Figure 16A-B shows induction of MEKKCOOH expression by IPTG in Swiss 3T3 cells increases the number of condensed cells and stimulates c-Myc transactivation. In [panel A] Figure 16A, cells were incubated in the presence or absence of 5 mM IPTG for forty eight hours. Cells were stained with acrodine orange and condensed cells quantitated per 1000 cells counted per coverslip. In [panel B] Figure 16B Swiss 3T3 cells with inducible MEKKCOOH were incubated in the presence or absence of IPTG. The indicated cells were then exposed to UV-C irradiation and then fixed and stained with propidium iodide. The percentage of apoptotic cells was enumerated.

The paragraph beginning at page 9, line 1, has been replaced with the following rewritten paragraph:

[Figure 17] Figure 17A-B shows that MEKKCOOH stimulates JNK/SAPKm but did not activate ERK (p42/44 MAPK) or p38Hog1. Induction of MEKKCOOH does not activate ERK (Figure 17A) or p38 (Figure 17B), whereas PDGF or sorbitol, (used as

positive controls) do. Activation of the cells with PDGF or sorbitol activated ERK and p38/Hog1 as a control.

The paragraph beginning at page 9, line 5, has been replaced with the following rewritten paragraph:

[Figure 18] Figure 18A-B shows that induction of MEKKCOOH expression did not significantly increase Gal4/Jun transactivation [(left panel)] (Figure 18A). Transient transfection of MEKKCOOH resulted in increased Gal4/Jun transactivation in the MEKK2 Swiss 3T3 clone [(right panel)] Figure 18B.

The paragraph beginning at page 9, line 8, has been replaced with the following rewritten paragraph:

[Figure 19] Figure 19A-B shows that competitive inhibitory JNK/SAPK(APF) attenuates GAl4/Jun but not Gal4/myc activation. The results are representative of three independent experiments where a three-fold excess of JNK/SAPK(APF) inhibited approximately 65% of Gal4/Jun activation (Figure 19A) with no effect on Gal4/myc activation (Figure 19B).

The paragraph beginning at page 9, line 20, has been replaced with the following rewritten paragraph:

[Figure 24 This figure] Figure 24A-B shows that TNF induces apoptosis in L929 cells and that this effect is blocked by bFGF. In [panel A] Figure 24A cells were treated with the indicated concentrations of TNFα for 15hours and were assayed for uptake of neutral red. In [panel B] Figure 24B cells were untreated (solid bars), treated with 0.5 ng/ml bFGF (dotted bars) or 5.0 ng/ml bFGF (hatched bars) and the indicated concentrations of TNFα for 18 hours. Cell viability was assessed by neutral red assay.

The paragraph beginning at page 9, line 26, has been replaced with the following rewritten paragraph:

[Figure 25] Figure 25A-B shows the activation of [JNK and] MAPK in L929 cells. [In panel A cells were treated for 10 minutes with the indicated concentration of TNFa. JNK activation was measured using a solid phase kinase assay resulting in phosphorylation of GST-Jun.] In [panel C] Figure 25A the time course of MAPK activation is shown. MAPK was isolated from cell lysates on DEAE sephacel columns and MAPK activation was measured by phosphorylation of the EGFR peptide substrate. [Panel C] Figure 25B depicts the concentration curve of MAPK activation by TNFα. Cells were treated with the indicated concentration of TNFa and MAPK was assayed.

The paragraph beginning at page 9, line 35, has been replaced with the following rewritten paragraph:

[Figure 27] Figure 27A-B shows that bFGF does not inhibit TNF□ stimulation of JNK activity. In [panel A] Figure 27A serum starved L929 cells were treated as indicated. Radiolabel incorporated into GST-Jun is expressed in arbitrary phosphorimaging units. In [panel B] Figure 25B cells were stimulated as indicated and assayed for MAPK activity.

The paragraph beginning at page 10, line 3, has been replaced with the following rewritten paragraph:

[Figure 28] Figure 28A-B shows the effect of dominant negative N17 Ras or constitutively active V12 Ras on MAPK and JNK activities. In [panel A] Figure 28A cells were uninduced (-) or induced (+) to express N17 Ras by overnight treatment with 5 mM IPTG. The cells were unstimulated(-) or stimulated(+) for 10 min with 0.5ng/ml

bFGF. MAPK activity was assayed. In [panel B] Figure 28B 41.LAC1 or V12 Ras cells were induced with IPTG, stimulated as indicated and analyzed for MAPK activation.

The paragraph beginning at page 10, line 15, has been replaced with the following rewritten paragraph:

[Figure 30] Figure 30A-B shows the inhibition of MAPK activity and elimination of the bFGF protective effect of treatment with the MEK-1 inhibitor PD #098059. In [panel A] Figure 30A serum starved L929 cells were untreated or treated for 1 hour at 37°C with the MEK-1 inhibitor (PD) and then unstimulated or stimulated with bFGF. MAPK activity was measured. In [panel B] Figure 30B L929 cells were untreated or treated for 1 hour at 37°C with PD and then were untreated or treated with TNFα alone or in combination with bFGF for 18 hours. Cell viability was assessed by neutral red assay.

The paragraph beginning at page 95, line 5, has been replaced with the following rewritten paragraph:

The transfected cells were incubated overnight and then lysed using methods standard in the art. The luciferase activity of each cell lysate was measure on a luminometer. The results shown in Figure 13 indicate that MEKK is selectively capable of stimulating the phosphorylation of c-Myc transactivation domain in such a manner that the c-Myc domain is activated and induces transcription of the transfected luciferase gene. In addition, the results indicate that MEKK does not stimulate CREB activation. Also, activated Raf is unable to stimulate Myc activation. A schematic representation of the activation mechanism of c-Myc protein by MEKK is shown in [Figure 14] Figure 14A-B.

The paragraph beginning at page 102, line 25, has been replaced with the following rewritten paragraph:

It was found that IPTG-induced $MEKK_{COOH}$ expression stimulated signal transduction pathways that made the cells significantly more sensitive to stresses that induce cell death. For example, cells expressing $MEKK_{COOH}$ were highly sensitive to ultraviolet irradiation. Two hours after exposure to ultraviolet irradiation greater than 30% of the $MEKK_{COOH}$ expressing cells became morphologically highly condensed and appeared apoptotic. In contrast, the population of uninduced cells showed no increase in condensed apoptotic-like cells at this time point [(Figure 16)] Figure 16A-B. Thus, overnight induction of $MEKK_{COOH}$ expression modestly increased the basal index of morphologically condensed cells and primed the cells for apoptosis in response to UV irradiation. The results indicate that MEKK-regulated signal transduction pathways enhance apoptotic responses to external stimuli.

The paragraph beginning at page 103, line 1, has been replaced with the following rewritten paragraph:

C. Expression of $MEKK_{COOH}$ stimulates JNK/SAPK and the transactivation of c-Myc and Elk-1 The ability of $MEKK_{COOH}$ but not BxBRaf expression to induce cell death indicates that each kinase regulates different sequential protein kinase pathways. Cells were incubated for 17 hours in the absence or presence of IPTG and assayed for JNK/SAPK activity. The induction of $MEKK_{COOH}$ expression in Swiss 3T3 cells, as predicted, stimulated JNK/SAPK activity but did not activate either ERK or p38/Hog1 activity as shown in [Figures 17 and 18] Figures 17A-B and 18A-B. The results indicate that induction of MEKKCOOH results in the activation of JNK/SAPK which phosphorylates GST-c-Jun. Because known substrates for JNK/SAPK are transcription factors, we assayed $MEKK_{COOH}$ inducible clones for transactivation of specific gene

transcription. Chimeric transcription factors having the Gal4 DNA binding domain and the transactivation domain of c-Myc, Elk-1 or c-Jun were used for assay of $MEKK_{COOH}$ signaling using a Gal4 promoter-luciferase reporter gene (Hibi et al. *supra*; Sadowski, I et al. (1988) *Nature* 335:563-564; Gupta et al. *supra*; Marais et al. *supra.*). Surprisingly, IPTG-induced stable expression of $MEKK_{COOH}$ markedly activated the transactivation function of c-Myc and Elk-1 but had little effect on Gal4/Jun activity as illustrated in [Figure 18] Figure 18A-B. This result was unexpected since $MEKK_{COOH}$ transient expression stimulated Gal4/Jun activity, indicating that transient expression of $MEKK_{COOH}$ was capable of transactivating c-Jun function in Swiss 3T3 cells. In addition, the JNK/SAPK activity stimulated by IPTG-induction of $MEKK_{COOH}$ correlated with the characterized JNK/SAPK enzyme by fractionation on Mono Q FPLC. Thus, $MEKK_{COOH}$ expression in stable clones achieved with IPTG-induction selectively regulated Gal4/Myc and Gal4/E1k-1 but not Gal4/Jun even though JNK/SAPK was activated.

The paragraph beginning at page 104, line 5, has been replaced with the following rewritten paragraph:

To determine if JNK/SAPK activation was required for c-Myc transactivation in response to $MEKK_{COOH}$, Gal4/Myc activation was assayed in the presence or absence of JNK/SAPK(APF). The results are shown in [Figure 19] Figure 19A-B. The JNK/SAPK(APF) was used as a competitive inhibitor of JNK/SAPK for activation by the immediate upstream JNK kinase/SEK-1 enzyme (Kyriakis et al. *supra*; Sluss, et al (1994). *Mol Cell. Biol.* 14:8376-8384; Lin et al (1994) *Science* 268:286-290; Sanchez et al. (1994) *Nature* 372:794-800). In transient transfection assays, expression of JNK/SAPK(APF) inhibited approximately 65% of the Gal4/Jun activation in response to $MEKK_{COOH}$. In contrast, expression of JNK/SAPK(APF) had no effect on $MEKK_{COOH}$ activation of Gal4/Myc induction of luciferase activity. Thus, c-Jun transactivation

appears to be independent of the $MEKK_{COOH}$ stimulated pathway leading to c-Myc transactivation. Similarly, JNK/SAPK activation can be significantly inhibited with no effect on c-Myc transactivation.

The paragraph beginning at page 119, line 11, has been replaced with the following rewritten paragraph:

bFGF and TNFα independently regulate cytoplasmic protein kinase cascades [Fig. 27] Figure 27A-B demonstrates that 1 ng/ml TNFα has only modest stimulatory effects on MAPK activity [(panel B)] (Figure 27B) and 2.5 ng/ml bFGF has little or no effect on JNK activity [(Panel A)] Figure 27A. These concentrations of bFGF and TNFα give maximal activation of MAPK and JNK, respectively. Co-stimulation of L929 cells with bFGF, at concentrations that show partial protection against TNFα-mediated killing, did not alter the magnitude of JNK activation in response to TNFα. Similarly, co-stimulation of L929 cells with TNFα, at concentrations capable of causing cell death, had little or no effect on bFGF stimulation of MAPK activity [(Panel B)] Figure 27B. Thus, in relation to JNK and MAPK, TNFα and bFGF receptors independently regulate the activity of these two sequential protein kinase pathways in L929 cells.

The paragraph beginning at page 119, line 21, has been replaced with the following rewritten paragraph:

Inducible expression of inhibitory and activated Ras influences apoptosis Ras activation is required for many of the phenotypic responses resulting from the activation of tyrosine kinases. Signaling by the bFGF receptor involves several different effector pathways including Ras activation. To test the involvement of Ras in the bFGF protective response, the Lac Switch inducible expression system (see Methods) was used to control the expression of inhibitory N17 Ras and constitutively activated V12 Ras in L929 cells.

[Fig. 28] Figure 28A-B shows the functional consequence of expressing inhibitory N17 Ras or activated V12 Ras on MAPK [and JNK] activation in response to bFGF[and TNF α, respectively]. IPTG-regulated expression of the HA epitope-tagged Ras mutants (N17 and V12 Ras) [is shown in Panel D] was observed (data not shown). Expression of N17 Ras significantly blunted bFGF stimulation of MAPK [(Panel A)] Figure 28A, but had no effect on TNF stimulation of JNK [(Panel C)] (data not shown). With two independent clones, expression of V12 Ras did not constitutively activate the MAPK pathway, but did appear to enhance bFGF stimulation of MAPK [(Panel B)] Figure 25B. V12 Ras expression also had no effect on TNFα stimulation of JNK activity [(Panel C)] (data not shown). Similar results were found with independent L929 cell clones indicating the responses were the result of specific mutant Ras expression.